

An Investigation of Exosome Concentration and Exosomal microRNA (miR-155 and miR-222) Expression in Pregnant Women with Gestational Hypertension and Preeclampsia

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Introduction: Hypertensive disorders of pregnancy are characterized by widespread maternal endothelial dysfunction. Elevated secretion of exosomes has been associated with endothelial dysfunction. Exosomes play a role in cell–cell communication by transferring microRNAs. These microRNAs are associated with the pathogenesis of hypertensive disorders of pregnancy through the regulation of endothelial function. This study characterizes exosomes and determines exosomal miR-155 and miR-222 expression levels in women with gestational hypertension (GH) and preeclampsia (PE).

Methods: Exosomes were isolated and thereafter characterised using NTA, microscopy and ELISA. Results: Exosomes were elevated in the serum of pregnant women with GH and PE ($P < 0.05$). The circulating exosomes and placental exosomes were increased in both GH and PE ($P < 0.0001$). The exosomal miR-155 increased in PE but not in GH ($P < 0.05$). MiR-222 decreased in PE ($P < 0.05$).

Discussion: Elevated exosomes in pregnant women with GH and PE may be indicative of exosomes being potential biomarkers for both GH and PE. The difference in the exosomal miR-155 and miR-222 expression in PE and GH suggested that these two disorders have different pathological pathways.

Keywords: exosomes, gestational hypertension, MiR-155, MiR-222, preeclampsia

Introduction

Hypertensive disorders of pregnancy (HDP) are the leading cause of maternal and neonatal morbidity and mortality locally and globally¹. Approximately 18% of the global maternal deaths are due to HPDs.² In South Africa, approximately 14.8% of maternal deaths are due to HDP.¹ It is characterized by an aberrant increase in blood pressure with or without proteinuria in urine after the 20th week of gestation.³ Gestational hypertension (GH) and Preeclampsia (PE) are the most diagnosed hypertensive conditions in pregnancy.⁴ Both PE and GH are characterized by the development of new hypertension (systolic blood pressure of ≥ 140 mm Hg and diastolic blood pressure of ≥ 90 mm Hg) after 20 weeks of gestation.⁵ However, the difference pertains to comorbidities; women with PE have placentae characterized by high syncytial knots and different distributions of fibrin deposits.⁶ PE is also characterized by an increase in proteinuria (≥ 300 mg) and an increase in circulating extracellular vesicles (exosomes).

The etiology of HPD is still not well understood due to the multifactorial nature of the disorder. However, syncytiotrophoblast microparticles (STBM) directly released from the placenta into the maternal circulation were recently identified as factors that are antiangiogenic, pro-coagulant, and involved in endothelial dysfunction,⁷ a key pathological feature of HDP. Exosomes are a critical essential constituent of STBM. They are the smallest membrane vesicles (30–150 nm)⁸ released by exocytosis from multivesicular bodies of the endosome. Exosomes are secreted by multiple cell types and can be found in different body fluids such as saliva, plasma and urine.⁹ They have emerged as

critical biological signalling entities because their cargo contains important biological molecules such as proteins, mRNA, and miRNA to induce cell-to-cell communication and signalling throughout the body. Therefore, it may play a vital role in the pathogenesis of diseases.

The biogenesis, characterization, and function of exosomes are new areas of study that has sparked considerable interest in the last decade. Exosomes (along with other types of extracellular vesicles) play a vital role in the regulation of a wide range of physiological and pathological cellular processes. They may be utilized for therapeutic purposes and act as candidate biomarkers in various diseases.¹⁰ Recently, exosomes have been linked to the pathogenesis of PE,⁷ but little is known about GH.

The release of exosomes is dependent on their microenvironment and is influenced by several factors, including oxygen tension and glucose concentrations.¹¹ However, the role of exosomes in the pathophysiology of PE and GH is not well understood. It has been reported that the total and placental exosomes with no known associated pathologies play a pivotal role in the regulation of endothelial cell migration.¹² Under normal conditions, maternal circulating placental exosomes increase with gestational age¹², this inversely correlates to endothelial cell migration. Conversely, it has been demonstrated that placental exosomes could play a pathological role in PE.⁷ Reports also indicate that exosomes regulate immune tolerance during normal and complicated pregnancies.^{13,14} In addition, exosomal miRNAs can be incorporated by the recipient cells, where they modulate functional effects by changing host gene expression.¹⁵

MicroRNAs have been associated with a wide range of physiological processes, including proliferation, differentiation, apoptosis, inflammation, and angiogenesis.¹⁶ These processes are disrupted in HDP, suggesting that miRNAs play a crucial role in HDP pathogenesis. MiR-155 are essential regulators of eNOS expression, and inhibition of miR-155 restores eNOS expression and improves endothelial dysfunction.¹⁷ The majority of NO in the circulation is synthesized from the endothelium, aberrant expression of eNOS may reduce VEGF expression, causing endothelial dysfunction¹⁸. MiR-222 is one of the miRNAs that play a role in the pathogenesis of GH and PE.¹⁹ Upregulation of miRNA-222 is associated with the increased estradiol secretion and the early phases of pregnancy development are thought to depend on an elevation of oestradiol secretion.²⁰

There is a lack of information regarding the role of exosomes in GH and PE. These disorders are classified as part of HDP and are characterized by widespread endothelial dysfunction.²¹ In addition, some women with GH are reported to develop PE in their third trimester of the subsequent pregnancy.²² Therefore, there is a possible link between these two disorders. Hence, the present study investigated the association of exosomes in the pathogenesis of GH and PE. The study aimed to determine the role of exosomes and exosomal miRNAs (miR-155 and miR-222) expression in GH and PE.

Methods and Materials

Regulatory Permissions

Ethical approval was received from the University of KwaZulu-Natal Biomedical Research Ethics Committee (BE229/16), South Africa. Health authority permission was also obtained, and all research participants provided written informed consent. The research was conducted ethically according to the principles of the Declaration of Helsinki.

Study Population

This is a cross-sectional study, and participants were recruited at a regional hospital in Durban, South Africa. Participants included normotensives (NH) with no obstetrical or medical complications ($n = 15$), GH ($n = 15$) and PE ($n = 15$). All participants were aged between 17 and 45 years. Patients with chronic hypertension, previous history of PE, diabetes, and other cardiovascular disorders were excluded. GH was defined as a blood pressure of $\geq 140/90$ mmHg with no proteinuria.²³ Late-onset PE was defined as a sustained blood pressure of $\geq 140/90$ mmHg with proteinuria of at least 300 mg after 34 weeks of pregnancy.¹

Sample Collection

Blood samples were collected [BD Vacutainer Tubes (EDTA), Becton Dickinson and Company, South Africa], and the serum samples were stored at -80°C for analyses.

Isolation and Purification of Exosomes from Maternal Circulation

Exosomes were isolated according to the method described previously in our laboratory.⁷ Serum (1 mL) was diluted with an equal volume of phosphate buffered saline (PBS; pH 7.4). Exosomes were isolated and purified by differential ultracentrifugation using a 30% sucrose cushion. In brief, to remove cells, centrifugation was initially performed at 2000 g at 4°C for 30 min, followed by 12,000 g at 4°C for 45 min. To remove the remaining debris, the supernatant was transferred to other centrifuge tubes and centrifuged at 110 000 g at 4°C for 120 min (Optima™ MAX-XP Ultracentrifuge, fixed angle MLA-55 rotor, Beckman Coulter Inc., Brea, CA, USA). To remove particles that were larger than 200 nm, the pellet was suspended in 2 mL of PBS and filtered through a 0.20 μm pore filter (Cellulose acetate, GVS™, Europe). The filtrate was centrifuged at 110 000 g at 4°C for 70 min following which the pellet was re-suspended in 2 mL PBS (pH 7.4) and centrifuged at 110 000 g for 70 min at 4°C . To purify the exosomes, the exosome pellet was suspended in 2 mL of PBS and subsequently purified using a 30% sucrose cushion and centrifuged at 110 000 g at 4°C for 75 min. The final pellet was re-suspended in 300 μL of PBS and stored at -80°C .

Nanoparticle Tracking Analysis

The size distribution of exosomes and their concentration were determined using the NS500 equipped with a 405nm laser and sCMOS camera (NanoSight NTA 3.0 Nanoparticle Tracking and Analysis Release, Version Build 0069). Serum samples were diluted with PBS (1:100) prior to analysis in order to obtain particle distribution of 10 and 100 particles per image (optimal, 50 particles per image) before the analysis with NTA system. Samples were introduced into the sample chamber using the following script: PUMLOAD, REPEATSTART, PRIME, DELAY 10, CAPTURE 60 and REPEAT 5. Videos were recorded at a camera level of 10, camera shutter speed of 20ms and camera gain of 600, these settings were kept constant between samples. Each video was then analysed to give the mean particle size together with the concentration of particles. The size of the exosomes was represented as the mean particle size \pm SEM.

Transmission Electron Microscopy

Exosomes were applied to a continuous carbon grid and negatively stained with 2% uranyl acetate. The size and morphology of the particles were examined using a JEOL 1010 transmission electron microscope (JEOL, Peabody, MA, USA) at the Microscopy and Microanalysis Unit, University of KwaZulu-Natal.

Quantification of the Circulating Exosomes

The concentration of total exosomes in the circulation was determined by the quantification of total immune-reactive CD63 enzyme-linked immune absorbency assay (ExoELISA™, System Biosciences, Mountain View, CA), as described by.^{7,11} CD63 is not an exosome specific marker but is commonly bound to the exosomal membrane and hence the method employed used isolated and purified exosomes, which contain the CD63 marker. The kit used, consists of an exosome specific primary CD63 antibody developed by the manufacturer. Briefly, exosomes were immobilized on microtiter plates for overnight at 37°C using exosome binding buffer supplied the manufacturer (System Biosciences). Plates were washed and incubated at room temperature for 1h with exosome specific primary antibody (CD63), followed by a wash step and incubation with secondary antibody (1:5000) at RT for 1h with agitation. Plates were thereafter washed and incubated with Super-sensitive TMB ELISA substrate at RT for 45 min with agitation. The reaction was terminated using Stop Buffer solution. Absorbance was measured at 450nm. The number of exosomes/mL were obtained using an exosomal CD63 standard curve that was generated using the calibrated exosome standard that was supplied.

Quantification of Placental Derived Exosomes

The relative concentration of placental-derived exosomes was determined by the quantification of human placental alkaline phosphatase in the exosomal fraction using a commercial ELISA kit (Elabscience, E-EL-H1976, Wuhan, P.R.C), as described by.^{7,11} Briefly, exosomes were allowed to bind to the primary PLAP specific antibody coated plates by incubation at 37°C for 90 min. Plates were washed and 50 µL of HRP-conjugate was added to each well and incubated at 37°C for 20 min. Plates were washed and incubated with 50 µL of substrate A and 50 µL of substrate B at 37°C for 15 min. The incubation was terminated using 50 µL of stop solution at RT for 2 min under agitation. Absorbance was measured at 450 nm. Exosomal PLAP was expressed as pg/mL serum. The quantification of PLAP in the exosomal fraction indicates the relative concentration of placental-derived exosomes (PLAP exosomes) in maternal circulation.

RNA Isolation

RNA was isolated from 100µL exosome sample using the PureLink RNA isolation Kit according to the manufacturer's instructions (ThermoFisher Scientific) and was eluted in 50µL of nuclease free water.

Quantitative Real-Time Polymerase Chain Reaction Analysis for miRNAs

Total RNA from each sample was reverse transcribed into cDNA using iScript cDNA Synthesis Kit according to manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, USA). Quantification of miRNAs was done using SYBR-Green 1 master mix with the light cycler 96 (Roche). 2µL of cDNA was used for qRT-PCR. The master mix contained 10µL SYBER- Green, 2µL forward respectively and 4µL RNase-free water for a final volume of 20µL. All samples were run in duplicates. The 3-step amplification procedure was performed at 95°C for 15 min, 60°C for 30 secs, and 72°C for 30 sec. The U6 small nuclear RNA (RNU6) was used to determine relative miRNA expression following a protocol by.²⁴ The miRNAs of interest were miR-222 and miR-155 (Table 1).

Statistical Analysis

GraphPad Prism 5 was used for statistical analysis. Statistical analysis was performed using one-way analysis of variance (ANOVA). Results were expressed as mean ± SEM from 15 patients in each group. P values ≤0.05 were considered as significant.

Results

Exosome Isolation and Characterization

Figure 1 shows the quantity of total exosome concentration in serum of normotensive, GH and PE groups obtained from the nanoparticle tracking analysis (NTA). There was a significant increase in the total exosome concentration of GH ($6.61 \pm 0.921 \times 10^8$ *total exosome/ml*) and PE group ($8.06 \pm 0.877 \times 10^8$ *total exosome/ml*) as compared to normotensive group ($2.63 \pm 0.067 \times 10^8$ *total exosome/ml*, $P < 0.05$). Particles of ~ 100 nm diameter were obtained from the TEM (Figure 2). In addition, CD63 ELISA demonstrated a significant increase in CD63 protein of both GH group ($2.59 \pm 0.65 \times 10^8$ *total exosome/ml*) and of PE group ($5.19 \pm 1.04 \times 10^8$ *total exosome/ml*) compared to normotensive ($5.60 \pm 1.52 \times 10^7$ *total exosome/ml*, $P < 0.001$) (Figure 3).

Table 1 MicroRNA Primer Sequence

MicroRNAs	Forward Primer	Reverse Primer
miR-155	CTGTTAATGCTAATCGTGATAG	GCAGGGTCCGAGGT
miR-222	TCCAGTGCAGGGTCCGAGGTAT	TAATAGAAAGCTACATCTGGCTACTGGGT

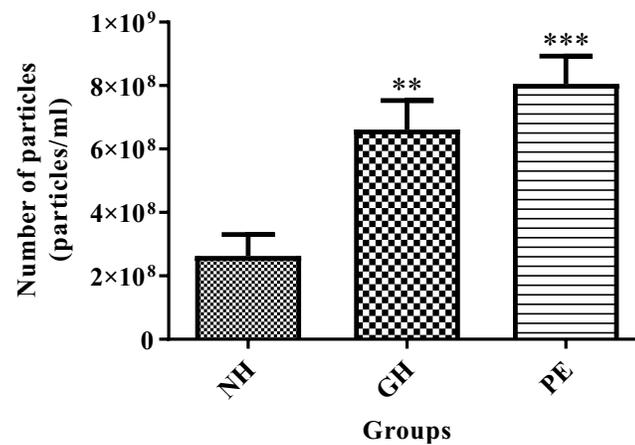


Figure 1 Total exosome concentration in serum of normotensive (NH), gestational hypertension (GH) and preeclampsia (PE) patient groups. **NH vs GH, ($P < 0.01$) and ***NH vs PE ($P < 0.001$). **and ***Shows the level of significance between groups.

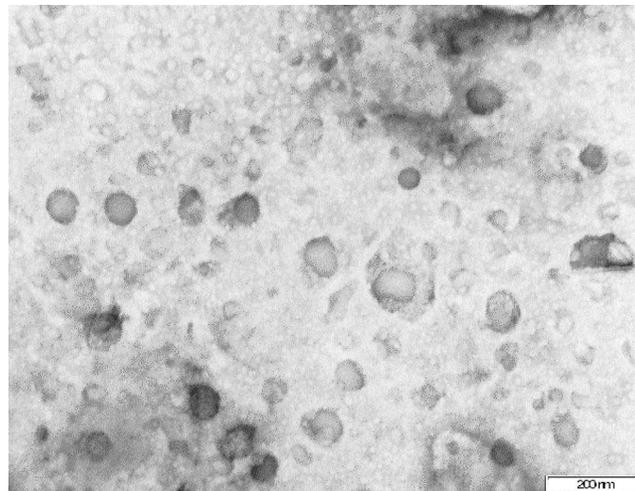


Figure 2 Electron micrograph of isolated exosomes, scale bar 200 nm.

Quantification and Contribution of Placental-Derived Exosomes to Total Exosomes from Maternal Serum

To determine the relative contribution of placental exosomes to total exosomes present in maternal serum, the PLAP content per exosome was determined. The concentration of PLAP⁺ exosomes in maternal serum is represented in Figure 4. There was a significant increase in PLAP⁺ exosome concentration in both GH group (174.8 ± 20.66 pg/mL) and PE group (255.4 ± 16.76 pg/mL) as compared with normotensive (28.48 ± 6.19 pg/mL, $P < 0.001$).

MiRNA Expression in GH, PE and Normotensives

Figures 5 and 6 show exosomal miRNAs expression in normotensive (NH), PE and GH sample groups. There was a significant increase of exosomal miR-155 expression in PE as compared to normotensive exosomes ($P < 0.05$) (Figure 5). There was a significant decrease in miR-222 expression in PE as compared with normotensive exosomes ($P < 0.05$) (Figure 6).

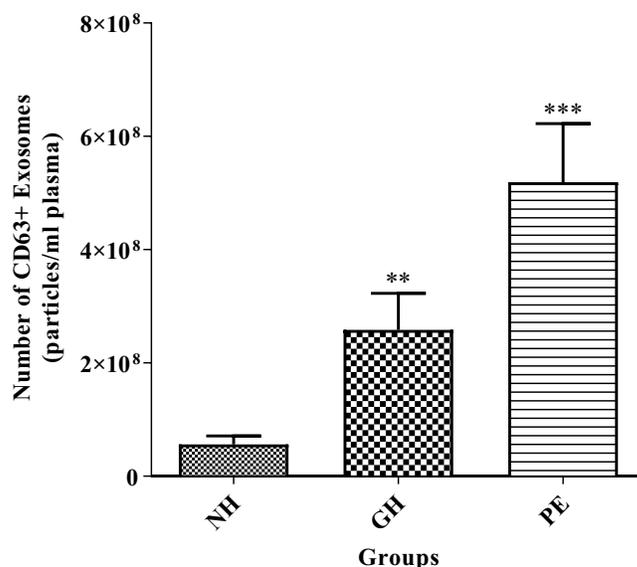


Figure 3 Total exosome concentration (CD63) in serum of normotensive (NH) and gestational hypertension (GH) and preeclampsia (PE) patient groups. **NH vs GH, ($P < 0.01$) and ***NH vs PE ($P < 0.001$). **and ***Shows the level of significance between groups.

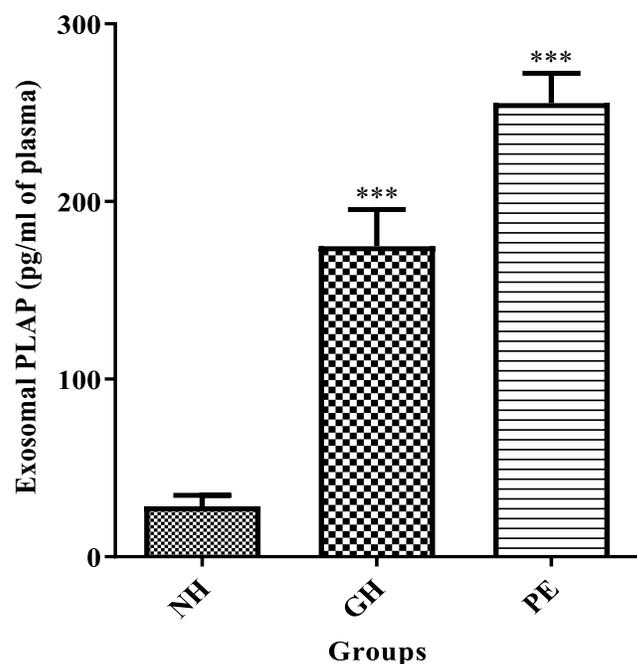


Figure 4 The concentration of placental derived exosomes (PLAP⁺ exosomes) in maternal serum. ***NH vs GH and ***NH vs PE, ($P < 0.001$). ***Shows the level of significance between groups.

Discussion

In the present study, the levels of total circulating exosomes in the serum of GH and PE were elevated (vs normotensive patients) (Figure 1). This study is in accordance with our previous findings of an increase in the total exosomes in PE (vs normotensives).⁷ The current findings add new information supporting exosomes as potential biomarkers of GH. The similarity in findings is not surprising given the linked pathologies involving endothelial dysfunction.²¹ This cellular stress in the form of endothelial dysfunction may stimulate secretion of exosomes²⁵ and the exosomes themselves may cause endothelial dysfunction.

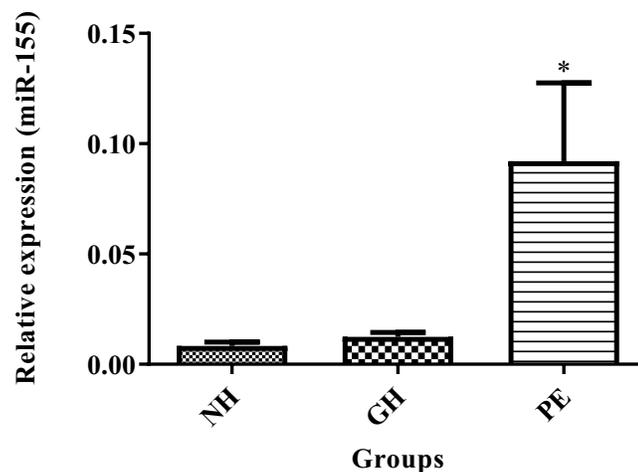


Figure 5 Exosomal miR-155 expression levels in normotensive (NH), PE and GH patient groups. *NH vs PE, ($P < 0.05$). *Shows the level of significance between groups.

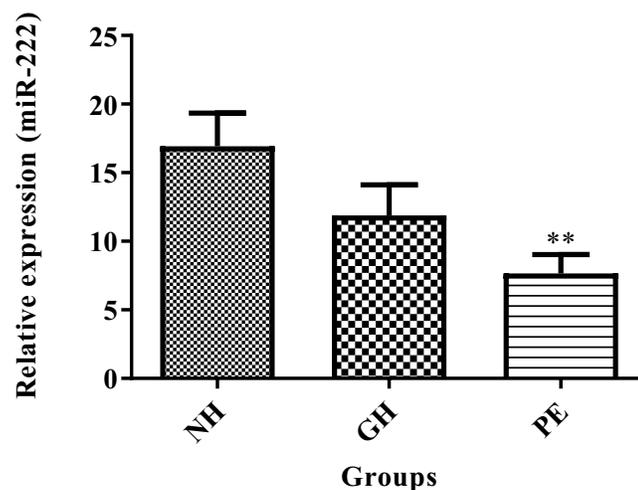


Figure 6 Exosomal miR-222 expression levels in normotensive (NH), PE and GH patient groups. **NH vs PE, ($P < 0.01$). **Shows the level of significance between groups.

Characterization of the isolated particles using TEM was positive for exosomes as it showed particle size range of exosomes (75nm – 107nm) (Figure 2). A significant increase in CD63 in GH and PE as compared to normotensive exosomes was observed (Figure 3), confirming the presence of exosomes in the serum. Furthermore, placental exosome levels were significantly increased in GH and PE as compared to normotensives (Figure 4). Thus, the study showed that exosomes are not only potential biomarkers of PE but also of GH.

Altered expression of microRNAs may play a role in the pathogenesis of PE.^{19,26} Importantly, it has been reported that majority of miRNAs in serum are detectable in exosomes²⁷ and exosome function as intercellular communicators via transfer of the biological information such as miRNAs. Leukemia cells communicate to endothelial cells via exosomal miRNAs which enhances endothelial cell migration²⁷ thus exosomal miRNAs are very important for endothelial cell communication.

We found significantly increased levels of circulating exosomal miR-155 expression in PE (Figure 5). These findings correlate with previous study that found increased expression of miR-155 in placentae of PE patients¹⁷ and suppression of eNOS expression in trophoblastic cells, possibly impacting on their migratory behaviour. However, further work need to check if these were of placental origin. It is well known that nitric oxide is a potent vasodilator and decreased

bioavailability leads to endothelial dysfunction. The eNOS expression is modulated via the modification of eNOS mRNA stability by affecting the binding of some cytoplasmic proteins with eNOS mRNA 3' untranslated regions. MiR-155 inhibits eNOS mRNA stability by binding 3'UTR thus downregulating eNOS expression.¹⁸ The increased exosomal miR-155 in PE may play a role in the endothelial cells' eNOS suppression, thus leading to endothelial dysfunction. In contrast, we found no significant difference between miR-155 expressions in GH vs normotensives. This may be due to the differential in pathological pathways of these two disorders, particularly the etiology.

We also found a significant decrease in miR-222 expression in PE compared to normotensive exosomes (Figure 6) but no significant difference between GH and normotensive exosomes. Upregulation of miR-222 increases oestradiol secretion.²⁰ It has been shown that miR-222 is downregulated in the placental syncytiotrophoblast vesicles (STBMs) of preeclamptic patients.²⁸

The MiR-221 and miR222 belong to the same family group and target common genes. They regulate endothelial cell migration and angiogenesis by targeting stem cell factor (SCF) receptor. SCF plays a vital role in promoting the survival and migration of endothelial cells. Thus, downregulation of miR-222 affects SCF and may lead to poor endothelial migration.²⁹ In addition, miR-222 has also been found to induce the production of endothelial nitric oxide.³⁰ Therefore, downregulation of miR-222 reduces nitric oxide bioavailability. Thus, this miRNA may play a pivotal role in the pathogenesis of PE.

Limitations

The eNOS expression in the exosomes was not measured.

Conclusion

Our preliminary studies support the role of exosomes as potential biomarkers of both PE and GH. Exosomal miRNA was altered in PE, keeping with the known pathology of the disease. In GH, we found no alteration of the investigated miRNAs. The differential findings are in keeping with the fact that the pathology is different with some common features. The clinical utility is based on the fact it will aid in diagnostics.

Ethics

Ethical approval for this study was obtained from the University of KwaZulu-Natal Biomedical Research Ethics Committee on 27/07/16. The BREC reference number allocated to this study is BE229/16.

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Disclosure

The authors of this study declare that there are no conflicts of interest.

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